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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

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Date of mailing (day/month/year) 21 September 1998 (21.09.98)	
International application No. PCT/US98/01322	Applicant's or agent's file reference 50015/013WO1
International filing date (day/month/year) 22 January 1998 (22.01.98)	Priority date (day/month/year) 22 January 1997 (22.01.97)
Applicant RUBIN, Lee, L. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
13 August 1998 (13.08.98)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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IN VIVO APOPTOSIS SCREENING

Background of the Invention

5 This invention relates to apoptosis, which is associated with physiological or programmed cell death (PCD). Apoptosis occurs in embryonic development, hormone deprivation of endocrine or other hormone-dependent or sensitive cells, cells responding to mild thermal or metabolic stress, and normal tissue turnover. Compounds which affect PCD (either
10 accelerating or inhibiting the process) are potentially useful as therapeutics to treat a wide range of medical disorders, including cancer, AIDS, autoimmune disorders such as rheumatoid arthritis, and neurodegenerative diseases such as multiple sclerosis.

 Cell death in many types of cells including neurons can be thought of
15 as a three-step process. The first step is the transmission of information about the status of the cell from outside the cell to the cytoplasm, or from the cell membrane to the nucleus. This information may follow the appearance of an apoptosis-inducing factor such as the Fas ligand, or the disappearance of a survival-promoting factor, such as nerve growth factor (for some types of
20 neurons). The second step is gene transcription and translation into protein. This second step can be blocked by compounds such as actinomycin D or cycloheximide, compounds which block transcription or translation. In this manner, these agents block some types of apoptosis. In the third step, the effectors of cell death are activated; these effectors include (in many types of
25 cells) cysteine proteases which cleave after aspartic acid residues; these agents

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are now termed caspases. Caspase inhibitors can interrupt the programmed cell death chain of processes, by blocking this third step.

Summary of the Invention

We have developed novel methods for screening potential apoptosis-
5 affecting compounds in an intact animal. One aspect of the invention features a method of testing a compound for the ability to affect cell death. This method includes: a) providing an osteichthes embryo which is translucent or transparent (i.e., optically clear), b) contacting the compound with the clear embryo, and c) visually observing the pattern or extent of cell death in the
10 embryo. The visual observation can be accomplished by a variety of methods known to those in the art of cytology, including labeling cells which undergo programmed cell death or apoptosis in the living embryo for visualization microscopically. For example, one method of labeling is carried out by terminal deoxynucleotide transferase dUTP nick labeling.

15 Another aspect of the invention features a method which includes the steps of: a) providing an animal (e.g., a vertebrate, such as a fish, and preferably a zebrafish, *Danio rerio*) which, at an embryonic stage, has been contacted with an agent which increases apoptosis in cells of the animal, b) contacting the animal with the test compound, and c) determining whether
20 the compound affects cell death in the animal. For example, the contacting step b) is carried out with the animal at an embryonic stage. The determining step c) may include determining whether the compound affects cell death in a Rohon-Beard neuron. The determining step c) may include determining whether the compound decreases or inhibits apoptosis (programmed cell death)
25 in a Rohon-Beard neuron that has been contacted with an agent which increases apoptosis. A compound which decreases or inhibits apoptosis is said to have

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rescued a cell. The determining step c) can also include using an antibody to label a cell, such as a Rohon-Beard cell, which undergoes cell death.

The invention also includes a test vertebrate embryo useful for screening compounds for the ability to affect cell death. The test embryo is prepared by a) providing a vertebrate embryo (e.g., a zebrafish), and b) contacting the embryo with an agent (e.g., stauro-sporine) which increases apoptosis in cells of the animal. This test embryo can be used in the methods disclosed herein for testing a compound for the ability to affect cell death.

Furthermore, the test embryo of the invention can also be used in a variety of methods for obtaining information on cellular processes. The cellular process which is investigated is selected from the group consisting of: a) neuronal cell function, b) neuronal connectivity, c) cell development, d) tissue development, and e) organ development. This method includes a) providing at least two embryos, namely a test zebrafish embryo and a control zebrafish embryo, b) subjecting a test embryo to test conditions, and c) visually observing differences in cells of the test and control embryos. The control embryo is treated such that the differences in visual observation result from application of the test conditions.

Test conditions include mutagenesis-inducing conditions (e.g., radiation or chemical agents), apoptosis-inducing or increasing agents, apoptosis-inhibiting or decreasing compounds, labeling or staining of one or more types of cells, marker compounds which label or indicate the present of a metabolite, and ligands for a receptor. In one embodiment, the test and control zebrafish embryos are pre-treated with an agent which affects programmed cell death. In another embodiment, the test conditions include a test compound (for example, either a cell death inhibitor, such as a caspase inhibitor, or a compound which increases cell death), and the observing step includes

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observing whether the test compound inhibits cell death in the test embryo, in other words, whether the test compounds rescues the test embryo. The agent can be a protein kinase inhibitor, such as staurosporine. The observing step can include observing neurons of the embryos for an interval of time sufficient to determine whether neurons in the test embryo which are saved from cell death develop or function normally. The observing step can include observing or comparing Rohon-Beard neurons in the test and control embryos. The invention also features a method for testing a compound for the ability to affect expression of a gene whose expression affects cell death, said method comprising the steps of: (a) providing an osteichthes test embryo which is translucent or transparent, wherein the gene is expressed in the embryo, wherein the gene either is not normally expressed in the osteichthes (embryo or adult), or is normally expressed in the osteichthes (embryo or adult) at a lower level than in the test embryo, (b) contacting the compound with the embryo, and (c) visually observing the pattern or extent of cell death in the embryo. The osteichthes embryo can be a zebrafish embryo. The gene can be a eukaryotic gene encoding a protein which inhibits cell death, such as a gene which encodes bcl-2, and is over-expressed in the embryo.

The invention in part is based on the observation that cells in the zebrafish embryo undergo apoptosis during normal development. According to the invention, the dying cells can be identified by simply viewing the whole live embryo (e.g., using Nomarski optics, or by a vital stain such as acridine orange). Alternatively, the dying cells can then be viewed and analyzed histologically, by staining the entire embryo using a method (the TUNEL method, explained in detail below), which detects DNA in the process of fragmenting during cell death. The pattern of TUNEL-positive cells at about 24 hours of development is easily discernable and very reproducible. The

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present studies have indicated that some of the dying cells are neurons, including Rohon-Beard sensory neurons, and thus the method provides an important tool for studying neuronal apoptosis.

5 Zebrafish are well suited for use in the method of the invention because of their rapid development, large brood size, external fertilization and, most importantly, the optical clarity of their embryos. Because the embryo is clear, apoptotic cells can be detected under the light microscope as highly refractive bodies, or stained using vital dyes such as acridine orange.

10 The amount of normal cell death in the developing fish embryo, as in other vertebrate embryos, is relatively small at any point in time. According to another aspect of the invention, the amount of cell death in these embryos can be greatly increased by briefly exposing the live embryos to a protein kinase inhibitor such as staurosporine. The experiments demonstrated that exposure of embryos to micromolar amounts of staurosporine produced large numbers of
15 TUNEL-positive cells. The number of dying cells was so large that they could easily be observed by microscopy, even without TUNEL staining. Cell death was so extensive that entire structures, e.g., the caudal tail region, were affected in a manner which could be observed microscopically.

20 Embryos can be treated with agents which inhibit apoptosis, e.g., caspase inhibitors, and the "saved" neurons can then be examined to determine whether they develop or function normally. Neural connectivity can also be observed using the system of the invention, as can cell, tissue, and embryonic development.

25 The invention can also be used (a) to test the effects of expression of a foreign gene whose expression ordinarily affects cell death, or (b) to screen for inhibitors of either a foreign gene whose expression ordinarily affects cell death or an endogenous gene that affects cell death. The method involves the

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steps of: (a) providing an osteichthes (preferably zebrafish) test embryo which is translucent or transparent, and in which the cell death-affecting gene is expressed; the gene either is one which is not normally expressed in the species of which the embryo is a member, or is normally expressed at lower levels, and is over-expressed in the test embryo. The test compound is contacted with the test embryo, and changes in the pattern or extent of cell death in the embryo brought about by the compound indicate its effect on cell death. Compounds which inhibit cell death-blocking compounds are useful, e.g., as anti-tumor adjuvant therapeutics.

Any of the known genes which express proteins which inhibit or accelerate cell death can be used; one example is the bcl-2 gene, the overexpression of which can be expected to block apoptosis. The embryo can be caused to express or over-express the cell death-affecting gene either via a transgenesis (the gene is inserted into embryo by standard microinjection techniques), or cells which have been transfected *ex-vivo* with the gene can be transplanted into the embryo. The human sequence for bcl-2 was published in Cleary, M.L. and Sklar, J., "Cloning and structural analysis of cDNAs for bcl-2 and hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation," Cell 47:19-28, 1986. The murine bcl-2 sequence was published in Negrini et al., "Molecular analysis of mbcl-2; Structure and expression of the murine gene homologous to the human gene involved in follicular lymphoma," Cell 49:455-463, 1987; and Nunez et al., "Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines," J. Immunol. 144:3602-3610, 1990.

The invention offers ease of use compared to other vertebrate embryonic systems (such as rodent or avian), while the process of cell death is similar or identical. Thus, compounds identified according to the invention

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which are effective anti-apoptotic agents are likely to be effective in mammalian systems as well. A further advantage of the invention is that the embryos are treated while they are still alive and developing, and it is therefore possible to determine if cells that are prevented from dying develop normally, an important consideration in screening anti-cell death drug candidates.

Other features and advantages of the invention will be apparent from the detailed description thereof, and from the claims.

Detailed Description of the Invention

Embryos

The embryos used in the methods of the invention preferably are clear and large enough for easy microscopic visualization. A number of osteichthes (bony fish) species are suitable, e.g., Medaka, Giant rerio. The preferred species is *Danio rerio*, the zebrafish, which has large, clear, easily-visualized embryos, and which reproduces in large numbers.

Cell Death Inhibition Assay

The assay is carried out as follows. Ten zebrafish embryos, at 90% epiboly (about 9 hours), are placed in a tank containing standard fish H₂O (60 mg Instant Ocean/liter distilled water) and varying concentrations (0.001-1000 μ M) of test compound. The embryos are incubated with the test compound overnight (until they reach about 22 hours of development), and examined *in vitro* and then prepared as follows for histologic examination.

The embryos are either acridine orange labelled or dechorionated and fixed in 4% paraformaldehyde made up in PBS. They can then be viewed with Nomarski optics or processed for TUNEL.

Acridine Orange Staining

Embryos of different ages are collected and incubated for 15-20 minutes in 5 μ g/ml acridine orange (Sigma). The embryos are then

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anaesthetized and observed under a microscope. Acridine orange-positive apoptotic cells are clearly visible under fluorescent illumination. They are then photographed and counted.

TUNEL Staining

5 The acronym TUNEL stands for terminal deoxynucleotide transferase (TdT) dUTP nick labelling, a method which detects DNA fragmentation which is characteristic of dying cells. In this method, terminal deoxynucleotide transferase DNA polymerases target the multitude of new 3'OH ends generated by DNA fragmentation in both early stage and
10 morphologically identifiable nuclei and apoptotic bodies. TdT polymerases add digoxigenin-dUTP to the 3'OH ends of the PCD fragmented DNA, which can then be detected by anti-digoxigenin alkaline phosphatase conjugate, and stained with substrate.

 In summary, the TUNEL staining method is carried out as follows:

15 Embryos are fixed and washed in PBT buffer. They are then treated with proteinase K, washed, and postfixed in paraformaldehyde. They are rinsed, fixed in methanol/acetic acid, rinsed again, and then subjected to the terminal transferase reaction. Embryos are incubated with terminal deoxytransferase (TdT) using reagents and conditions provided in the
20 Apoptosis Detection Kit supplied by Oncor, Inc. Enzyme incubation is overnight at 37°C . The reaction is stopped and the embryos are rinsed in PBT. For detection of labelled DNA, embryos are incubated in sheep anti-digoxigenin antibody conjugated to alkaline phosphatase. An alkaline phosphatase substrate X-phosphate/NBT in an appropriate buffer is added and
25 incubation continues for 15 minutes. The reaction is stopped, embryos are fixed, cleared, mounted, and viewed with Nomarski optics.

 The detailed TUNEL protocol is as follows:

Fixation

1. Dechorionate embryos and fix in 4% paraformaldehyde/PBS for 1 hour at room temperature. Wash 3 x 5 minutes in PBS. Embryos can be stored in methanol at 4°C overnight.

5 Permeabilization

1. Rehydrate by careful washes in 75% methanol + 25% PBT (1 x PBS, 0.1% Tween 70); 50% methanol; 50% PBT; 75% methanol; 75% PBT for 5 minutes each.

2. Wash 3 x for 5 minutes in PBT.

10 3. Incubate embryos in Proteinase K (10 µg/ml in PBS) at room temperature 20 minutes for post 16 hours. Wash 2 x for a few seconds in PBT.

4. Postfix embryos in 4% paraformaldehyde/PBS for 20 min. at room temperature.

5. Wash 5 x 5 minutes in PBT.

15 6. Postfix embryos for 10 minutes at -20°C with prechilled (-20°C) Ethanol:Acetate +2:1.

7. Wash 3 x 5 minutes in PBT at room temperature.

Terminal Transferase Reaction

20 1. Incubate embryos for 1 hour at room temperature in 75 µl (1 drop) equilibration buffer, reaction buffer and TdT enzyme are provided in the ApopTag In situ Apoptosis Detection Kit-Peroxidase, Oncor, Inc. For preparation of working strength TdT enzyme, mix the reaction buffer (S7105) with the TdT enzyme (S7107)=2:1 and add Triton X 100 to a final concentration of 0.3%).

25 2. Take off as much equilibration buffer as possible and add small volume of working strength TdT enzyme (The reaction worked already with as little as 17 µl working strength TdT enzyme). Incubate overnight at 37°C.

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Stop/Wash

1. Stop reaction by washing in working strength stop/wash buffer (prepare working strength stop/wash buffer by mixing 1 ml stop/wash buffer (S7100-4) with 17 ml distilled water) for 3 hours at 37°C.

5 2. Wash 3 x 5 minutes in PBT.

Detection

1. Block with 2 mg/ml BSA, 5% sheep serum in PBT for a minimum of 60 minutes (or use 4% BSA, 5% non-fat dry milk, 10% horse serum).

10 2. Incubate embryos for 2 hours at room temperature (or overnight at 4°C) in a 1/2000 dilution of preabsorbed sheep anti-digoxigenin-alkaline phosphatase conjugated Fab fragments.

3. Wash overnight with 2 mg/ml BSA in PBT with at least 4 changes of blocking buffer.

15 4. Equilibrate 3 x 5 minutes in freshly prepared NTMT buffer (0.1 M Tris-HCl pH 9.5; 50 mM MgCl₂; 0.1M NaCl; 0.1% Tween 20).

5. Perform color reaction with X-phosphate/NBT in NTMT on shaker in dark (4.5 µl of 75 mg/ml NBT in dimethylformamide and 3.5 µl of 50 mg/ml X-phosphate in dimethylformaldehyde in 1 ml NTMT buffer) for 15
20 minutes.

6. Stop reaction with washes in PBT.

Clearing and Mounting

1. Fix embryos in 4% paraformaldehyde/PBS for 30 minutes at room temperature.

25 2. Wash in PBT.

3. Clear and mount in glycerol - 30% - 50% - 70%.

4. Document with Nomarski optics.

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Other staining methods which detect DNA fragmentation characteristic of dying cells can be used in place of TUNEL staining, e.g., propidium iodide or Hoechst 33342 dye incubation followed by examination under fluorescence optics to detect condensed chromatin. Extraction of DNA is followed by resolution on gels to detect DNA "laddering" into nucleosome-sized fragments of about 180 bp.

Programmed Cell Death in Normal Embryos

TUNEL staining was used to identify the pattern of PCD occurring in various stages of normally-developing zebrafish. Embryos 30% epiboly to 24 hours were studied every hour, and embryos 24 hours to 48 hours were studied every two hours.

Diffuse PCD was observed in most regions during development, with concentrated regions of PCD which were localized spatially and temporally. The earliest PCD was detected in a few cells at 75% epiboly. Up to 12 somites, there is diffuse, seemingly random PCD, which then begins to concentrate toward the brain and tailbud. From 19 hours, a localized pattern of PCD was found in the lens and cornea of the eye, the otocyst, the cloacal opening, the olfactory placode, and portions of the nervous system, including the dorsoventral spinal cord.

Diffuse apoptosis is believed to occur amidst tightly packed cells in order to allow their free movement during periods of gradual morphogenesis. Highly localized concentrations of PCD may permit more radical morphogenesis. For example, localized cell death in the nervous system may clear the way for outgrowing axons; concentrated PCD in the olfactory placode coincides with the time that axons are exiting the placode and growing toward the telencephalon.

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PCD Increase with Kinase Inhibitors and Decrease with CaspaseInhibitors

The protein kinase inhibitor staurosporine is used at a concentration of between 10 μ M and 100 μ M. Embryos at the 22 hour stage are incubated for 120 minutes, washed in PBS, and either labelled with acridine orange or fixed and viewed in Nomarski optics or processed for TUNEL.

EXAMPLE 1

An assay according to the invention was carried out with staurosporine-pretreated zebrafish embryos, using, as a test compound, a tripeptide inhibitor of ICE-like proteases, carboxybenzoyl Val-Ala-Asp fluoromethylketone (zVADfmk). A control, carboxybenzoyl Phe-Ala-fluoromethylketone (zFAfmk), did not prevent apoptosis in either staurosporine-pretreated embryos, or untreated embryos. The results demonstrated that ICE-like proteases are likely to be involved in mediating apoptosis, within the nervous system and perhaps in other organ systems as well.

TABLE 1

Conditions	Normal PCD	Sts-induced PCD
untreated	25 \pm 3.15	455 \pm 132
+zVADfmk	10 \pm 2.76	31 \pm 3.47
+zFAfmk	31 \pm 2.79	322 \pm 64.27

Table 1. Number of TUNEL-positive cells in the dorsal half of 26-28 hour old zebrafish embryos spanning a 10-somite length. Values expressed as means \pm SEM of 5 embryos per condition.

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EXAMPLE 2

Rohon-Beard neurons are sensory neurons which undergo apoptosis. HNK-1 is a cell surface molecule which is predominantly expressed by Rohon-Beard neurons. Zebrafish embryos were double stained with TUNEL to mark those cells undergoing apoptosis and with HNK-1 antibody to identify Rohon-Beard neurons. Standard procedures were used for antibody labeling embryos ranging from 12 somites to 48 hours. Embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 4 hours; washed in PBS, incubated for 3 hours in PBS plus 3% goat serum and 0.1% Triton-X-100 (Sigma), and incubated overnight in a solution of monoclonal HNK-1 antibody (Sigma) diluted 1:1000. This was followed by washing in PBS plus 0.1% Triton (PBST) for at least one hour and incubating overnight in HRP-conjugated goat anti-mouse IgM diluted in PBST plus 1% goat serum. Embryos were washed as before and bound antibody was detected using diaminobenzidine (DAB) as the chromogen. Double labeling cells with HNK-1 antibody and TUNEL involved first HNK-1 antibody staining followed by TUNEL using the methods sequentially as already described.

Color photographs of zebrafish spinal cord demonstrated that, in an apoptosis assay, Rohon-Beard neurons were rescued by an apoptosis inhibitor compound such as zVADfmk (TUNEL-negative). In contrast, neurons not exposed to the inhibitor compound were TUNEL positive, indicating cell death. This example demonstrated that *in vivo* screening methods disclosed herein can reliably identify cells which are undergoing cell death, and, by substituting the known apoptosis inhibitor with a test compound or mixture of test compounds, also identify compounds which affect cell death, for example, apoptosis inhibitors.

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Other Embodiments

Based on the description and examples above, and the claims below, the essential features and advantages of the present invention can be ascertained. Without departing from the spirit and scope of this disclosure, 5 further various modifications or substitutions can be made and are also within the invention.

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Claims

1. A test vertebrate embryo for screening compounds for the ability to affect cell death, said embryo being prepared by the process of:

a) providing a vertebrate embryo, and

5 b) contacting said embryo with an agent which increases apoptosis in cells of said animal.

2. The embryo of claim 1, wherein said agent is staurosporine.

3. The embryo of claim 1, wherein said vertebrate is a zebrafish.

4. A method of testing a compound for the ability to affect cell death, said method comprising the steps of:

10 a) providing an animal which, at an embryonic stage, has been contacted with an agent which increases apoptosis in cells of said animal,

b) contacting said animal with said compound, and

c) determining whether said compound affects cell death in said animal.

15 5. The method of claim 4, wherein step b) is carried out with said animal at an embryonic stage.

6. The method of claim 4, wherein said animal is a vertebrate.

7. The method of claim 6, wherein said vertebrate is a fish.

8. The method of claim 7, wherein said fish is a zebrafish.

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9. A method of claim 6, wherein said determining step c) includes determining whether said compound affects cell death in a Rohon-Beard neuron.

10. A method of claim 9, wherein said determining step c) further includes determining whether said compound rescues a Rohon-Beard neuron that has been contacted with an agent which increases apoptosis.

11. A method of claim 4, wherein said determining step c) includes using an antibody to label a cell which undergoes cell death.

12. The method of claim 4, wherein said agent is staurosporine.

13. A method of testing a compound for the ability to affect cell death, said method comprising the steps of:

- a) providing an osteichthes embryo which is translucent or transparent,
- b) contacting said compound with said embryo, and
- c) visually observing the pattern or extent of cell death in said embryo.

14. The method of claim 13, wherein said embryo is a zebrafish embryo.

15. The method of claim 13, wherein cells in said embryo undergoing programmed cell death are labeled in the living embryo for visualization microscopically.

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16. The method of claim 15, wherein labeling is carried out by terminal deoxynucleotide transferase dUTP nick labeling.

17. A method for obtaining information on cellular processes, said method comprising the steps of:

- 5 a) providing a test and a control zebrafish embryo,
 b) subjecting said test embryo to test conditions, and
 c) visually observing differences in cells of the test and control embryos,
said differences resulting from application of said test conditions.

10 18. The method of claim 17, wherein said test conditions include mutagenesis-inducing conditions.

19. The method of claim 17, wherein said test and control zebrafish embryos are pre-treated with an agent which affects programmed cell death.

15 20. The method of claim 17, wherein said test conditions include a test compound, and said observing step includes observing whether said test compound inhibits cell death in, or rescues, said test embryo.

21. The method of claim 20, wherein said observing step includes observing Rohon-Beard neurons.

22. The method of claim 19, wherein said agent is a protein kinase inhibitor.

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23. The method of claim 22, wherein said protein kinase inhibitor is staurosporine.

24. The method of claim 17, wherein said test conditions include contacting said embryos with a cell death inhibitor.

5 25. The method of claim 24, wherein said observing includes observing neurons of said embryos, to determine whether neurons in said test embryo which are saved from cell death develop or function normally.

26. The method of claim 24, wherein said cell death inhibitor is a caspase inhibitor.

10 27. The method of claim 17, wherein the cellular process which is investigated is selected from the group consisting of:

- a) neuronal cell function,
- b) neuronal connectivity,
- c) cell development,
- 15 d) tissue development, and
- e) organ development.

28. A method of testing a compound for the ability to affect expression of a gene whose expression affects cell death, said method comprising the steps of:

- (a) providing an osteichthes test embryo which is translucent or
- 20 transparent, wherein said gene is expressed in said embryo, wherein said gene

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either is not normally expressed in said osteichthes, or is normally expressed in said osteichthes at a lower level than in said test embryo,

(b) contacting said compound with said embryo, and

(c) visually observing the pattern or extent of cell death in said

5 embryo.

29. The method of claim 28, wherein said osteichthes embryo is a zebrafish embryo.

30. The method of claim 28, wherein said gene is a eukaryotic gene encoding a protein which inhibits cell death.

10 31. The method of claim 30 wherein said gene encodes bcl-2, and is over-expressed in said embryo.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01322

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 15/00; A61K 48/00

US CL :800/2; 424/9.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 424/9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	AMSTERDAM, A. et al. Requirements for Green Fluorescent Protein Detection in Transgenic Zebrafish Embryos. Gene. 1996, Vol. 173, pages 99-103, see especially page 102, col. 1, paragraph 3 and page 103, col. 1, paragraphs 2 and 3.	1-3 ----- 5-31
Y	MUNDLE, S.D. et al. Two <i>in situ</i> Labeling Techniques Reveal Different Patterns of DNA Fragmentation during Spontaneous Apoptosis <i>in Vivo</i> and Induced Apoptosis <i>in Vitro</i> . Anticancer Research. 1995, Vol. 15, pages 1895-1904, see especially abstract.	3-31



Further documents are listed in the continuation of Box C.



See patent family annex.

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
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Date of the actual completion of the international search

03 APRIL 1998

Date of mailing of the international search report

20 MAY 1998

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PATENT COOPERATION TREATY

REC'D 30 MAR 1999

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

3

Applicant's or agent's file reference 50015/013WO1	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US98/01322	International filing date (day/month/year) 22 JANUARY 1998	Priority date (day/month/year) 22 JANUARY 1997
International Patent Classification (IPC) or national classification and IPC IPC(6): C12N 5/00, 15/00; A61K 48/00 and US Cl.: 800/2; 424/9.2		
Applicant EISAI CO., LTD.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 13 AUGUST 1998	Date of completion of this report 16 MARCH 1999
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DEBORAH CROUCH, CHD.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US98/01322

I. Basis of the report

1. This report has been drawn on the basis of (*Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments*):

☒ the international application as originally filed.

☒ the description, pages 1-14, as originally filed.

pages NONE, filed with the demand.

pages NONE, filed with the letter of _____.

pages _____, filed with the letter of _____.

☒ the claims, Nos. 1-31, as originally filed.

Nos. NONE, as amended under Article 19.

Nos. NONE, filed with the demand.

Nos. NONE, filed with the letter of _____.

Nos. _____, filed with the letter of _____.

☒ the drawings, sheets/fig None, as originally filed.

sheets/fig NONE, filed with the demand.

sheets/fig NONE, filed with the letter of _____.

sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE.

☒ the claims, Nos. NONE.

☒ the drawings, sheets/fig NONE.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US98/01322

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims <u>1-31</u>	YES
	Claims <u>NONE</u>	NO
Inventive Step (IS)	Claims <u>NONE</u>	YES
	Claims <u>1-31</u>	NO
Industrial Applicability (IA)	Claims <u>1-31</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-31 the criteria set out in PCT Article 33(2) for novelty and (4) for industrial applicability.

Claims 1-31 lack an inventive step under PCT Article 33(3) as being obvious over Amsterdam et al (1996) Gene 173, 99-103 in view of Mundle et al (1995) Anticancer Research 15, 1895-1904 further in view of Illera et al (1993) Journal of Immunology, 151, 2965-2973. Amsterdam et al teach zebrafish embryos as an assay for determining expression levels of a DNA sequence encoding green fluorescent protein, and provides a discussion of promoter constructs needed for such expression (pages 100 and 102). Muddle et al teach TUNEL as a means to analyze for DNA fragmentation due to apoptosis (page 1898). Illera et al teach staurosporine as an apoptotic agent (2968 and 2969). Thus it would have been obvious to the ordinary artisan at the time of the instant invention to use zebrafish embryos in an assay to determine the apoptotic effect of agents such as staurosporine by DNA fragmentation analysis.

NEW CITATIONS

ILLERA, V.A. et al. Apoptosis in Splenic B Lymphocytes. Regulation by Protein Kinase C and IL-4. Journal of Immunology. 15 September 1993, Vol. 151, No. 6, pages 2965-2973, especially pages 2968-2971.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01322

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/00, 15/00; A61K 48/00

US CL : 800/2; 424/9.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 424/9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----	AMSTERDAM, A. et al. Requirements for Green Fluorescent Protein Detection in Transgenic Zebrafish Embryos. Gene. 1996, Vol. 173, pages 99-103, see especially page 102, col. 1, paragraph 3 and page 103, col. 1, paragraphs 2 and 3.	1-3 -----
Y		5-31
Y	MUNDLE, S.D. et al. Two <i>in situ</i> Labeling Techniques Reveal Different Patterns of DNA Fragmentation during Spontaneous Apoptosis <i>in Vivo</i> and Induced Apoptosis <i>in Vitro</i> . Anticancer Research. 1995, Vol. 15, pages 1895-1904, see especially abstract.	3-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

03 APRIL 1998

Date of mailing of the international search report

20 MAY 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DEBORAH CROUCH, PH.D.

Telephone No. (703) 308-0196

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/00, 15/00, A61K 48/00	A1	(11) International Publication Number: WO 98/31787 (43) International Publication Date: 23 July 1998 (23.07.98)
(21) International Application Number: PCT/US98/01322 (22) International Filing Date: 22 January 1998 (22.01.98)		(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(30) Priority Data: 9701245.4 22 January 1997 (22.01.97) GB (71) Applicant (for all designated States except US): EISAI CO., LTD. [JP/JP]; Tokkyobu Koishikawa, Bunkyo-ku 4-6-10, Tokyo 112-88 (JP). (72) Inventors; and (75) Inventors/Applicants (for US only): RUBIN, Lee, L. [US/US]; 2 Barnstable Road, Wellesley, MA 02181 (US). DE LEON GATCHALIAN, Christine [US/GB]; 17 Viscount Court, 1 Pembridge Villas, London W2 4AX (GB). (74) Agent: SHEN, Evelyn, D.; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-2214 (US).		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: <i>IN VIVO</i> APOPTOSIS SCREENING		
(57) Abstract A test animal for screening compounds for the ability to affect cell death, prepared by the process of: a) providing an animal embryo, b) contacting the embryo with a protein kinase inhibitor to increase apoptosis in cells of the animal; and methods of using the test animal.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
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CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: PAUL T. CLARK
CLARK & ELBING LLP
176 FEDERAL STREET
BOSTON, MASSACHUSETTS 02110-2214

COPY

WRITTEN OPINION

(PCT Rule 66)

DOCKETED FOR
ATTENTION

RECEIVED

NOV 09 1998

CLARK & ELBING

Date of Mailing
(day/month/year)

06 NOV 1998

Applicant's or agent's file reference

50015/013WO1

REPLY DUE

within TWO months
from the above date of mailing

International application No.

PCT/US98/01322

International filing date (day/month/year)

22 JANUARY 1998

Priority date (day/month/year)

22 JANUARY 1997

International Patent Classification (IPC) or both national classification and IPC
IPC(6): C12N 5/00, 15/00; A61K 48/00 and US Cl.: 800/2; 424/9.2

Applicant

EISAI CO., LTD.

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 22 MAY 2000

Name and mailing address of the IPEA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DEBORAH CROUCH, PH.D.

Telephone No. (703) 308-0196

JAB
for

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

☒ the international application as originally filed.

☒ the description, pages 1-14, as originally filed.
pages NONE, filed with the demand.
pages NONE, filed with the letter of _____

☒ the claims, Nos. 1-31, as originally filed.
Nos. NONE, as amended under Article 19.
Nos. NONE, filed with the demand.
Nos. NONE, filed with the letter of _____

☒ the drawings, sheets/~~fig~~ None, as originally filed.
sheets/~~fig~~ NONE, filed with the demand.
sheets/~~fig~~ NONE, filed with the letter of _____

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

3. ☐ This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box. Additional observations below (Rule 70.2(e)).

4. Additional observations, if necessary:

NONE

WRITTEN OPINION

International application No.

PCT/US98/01322

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims <u>1-31</u>	YES
	Claims <u>NONE</u>	NO
Inventive Step (IS)	Claims <u>NONE</u>	YES
	Claims <u>1-31</u>	NO
Industrial Applicability (IA)	Claims <u>1-31</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-31 the criteria set out in PCT Article 33(2) for novelty and (4) for industrial applicability.

Claims 1-31 lack an inventive step under PCT Article 33(3) as being obvious over Amsterdam et al (1996) Gene 173, 99-103 in view of Mundle et al (1995) Anticancer Research 15, 1895-1904 further in view of Illera et al (1993) Journal of Immunology, 151, 2965-2973. Amsterdam et al teach zebrafish embryos as an assay for determining expression levels of a DNA sequence encoding green fluorescent protein, and provides a discussion of promoter constructs needed for such expression (pages 100 and 102). Muddle et al teach TUNEL as a means to analyze for DNA fragmentation due to apoptosis (page 1898). Illera et al teach staurosporine as an apoptotic agent (2968 and 2969). Thus it would have been obvious to the ordinary artisan at the time of the instant invention to use zebrafish embryos in an assay to determine the apoptotic effect of agents such as staurosporine by DNA fragmentation analysis.

NEW CITATIONS

ILLERA, V.A. et al. Apoptosis in Splenic B Lymphocytes. Regulation by Protein Kinase C and IL-4. Journal of Immunology. 15 September 1993, Vol. 151, No. 6, pages 2965-2973, especially pages 2968-2971.

WRITTEN OPINION

International application No.

PCT/US98/01322

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: PAUL T. CLARK
CLARK & ELBING LLP
176 FEDERAL STREET
BOSTON, MASSACHUSETTS 02110-2214

APR 01 1999

COPY
PCT 7 19
QR

NOTIFICATION OF TRANSMITTAL OF
INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing
(day/month/year)

26 MAR 1999

Applicant's or agent's file reference

50015/013WO1

IMPORTANT NOTIFICATION

International application No.

PCT/US98/01322

International filing date (day/month/year)

22 JANUARY 1998

Priority Date (day/month/year)

22 JANUARY 1997

Applicant

EISAI CO., LTD.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized Officer

DEBORAH CROUCH, PH.D.

Telephone No. (703) 308-0196

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 50015/013WO1	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US98/01322	International filing date (<i>day/month/year</i>) 22 JANUARY 1998	Priority date (<i>day/month/year</i>) 22 JANUARY 1997
International Patent Classification (IPC) or national classification and IPC IPC(6): C12N 5/00, 15/00; A61K 48/00 and US Cl.: 800/2; 424/9.2		
Applicant EISAI CO., LTD.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

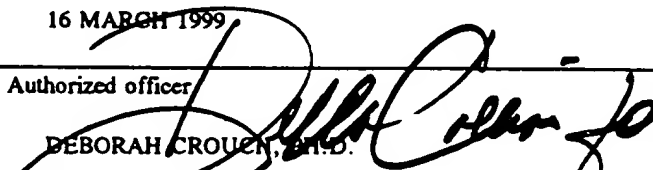
2. This REPORT consists of a total of 3 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
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- VIII ☐ Certain observations on the international application

Date of submission of the demand 13 AUGUST 1998	Date of completion of this report 16 MARCH 1999
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  DEBORAH CROUN, Ph.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US98/01322

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pages NONE , filed with the letter of _____.

pages _____ , filed with the letter of _____.

☒ the claims, Nos. 1-31 , as originally filed.

Nos. NONE , as amended under Article 19.

Nos. NONE , filed with the demand.

Nos. NONE , filed with the letter of _____.

Nos. _____ , filed with the letter of _____.

☒ the drawings, sheets/fig None , as originally filed.

sheets/fig NONE , filed with the demand.

sheets/fig NONE , filed with the letter of _____.

sheets/fig _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE .

☒ the claims, Nos. NONE .

☒ the drawings, sheets/fig NONE .

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US98/01322

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims <u>1-31</u>	YES
	Claims <u>NONE</u>	NO
Inventive Step (IS)	Claims <u>NONE</u>	YES
	Claims <u>1-31</u>	NO
Industrial Applicability (IA)	Claims <u>1-31</u>	YES
	Claims <u>NONE</u>	NO

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Claims 1-31 the criteria set out in PCT Article 33(2) for novelty and (4) for industrial applicability.

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